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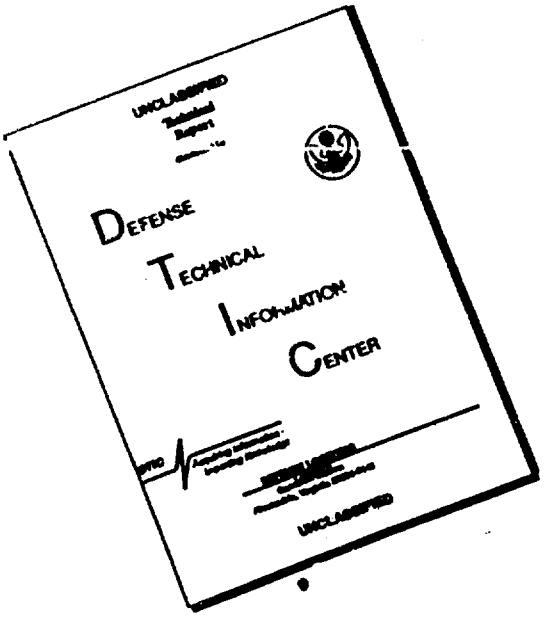

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Accelerated Detection of the Pathogens of Certain Diseases by the Fluorescent Antibody Method

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From the progressive methods of accelerated detection of agents in objects of the environment, laboratories in recent years have developed the method of fluorescent antibodies. The creation of the fluorochrome dyes, which are capable of combining chemically with the molecules of immune protein without disrupting their immunologic specificity, has opened up unlimited possibilities for the use of luminescent analysis in the area of microbiology.

The experimental studies of Soviet authors on the detection of the bacteria of the enteric group and of anthrax, and of foreign authors on the demonstration of the agent of melioidosis in the soil and in the diagnosis of influenza in patients, testify to the indisputable prospects of this method.

The present work was devoted to a study of the possibilities of hastening detection of the agents of several diseases, in material taken from environmental objects, with the aid of the fluorescent antibody method under the conditions prevailing in military bacteriological laboratories.

The work was carried out in such a way as to study the specificity and sensitivity of the method and the rapidity of investigation for the presence of known agents. We made use of our own laboratory specimens of typhoid fever, brucellosis, tularemia, anthrax, and normal sera labeled with the fluorescent dye (fluorescine-isocyanate and 1-dimethylaminonaphthalene-5-sulfochloride). The specificity of each serum had been demonstrated earlier in linear agglutination studies with homologous and heterologous antigens. This facilitated an increase in the objectivity of the results of the method of fluorescent antibodies, since in processing the latter we used the most highly specific sera, which did not react with heterologous antigens generally and which gave positive agglutinations in a dilution not greater than 1:40. The results of the study of the specificity of the method are shown in Table 1.

In order to exclude the possibility of nonspecific results associated with characteristics of the cultures, and in order to standardize the conditions of the experiments, we used as our homologous antigens the standard diagnostic antigens approved by the state control. The appropriate dilutions of these were prepared in distilled water, starting with the content of microbial bodies in each diagnostic sample.

Table 1

Results of processing with labelled sera

	typhoid						tularemia						brucellosis						anthrax						normal rabbit			normal horse		
	1			2			1			2			1			2			1			2								
	K	P	O	K	P	O	K	P	O	K	P	O	K	P	O	K	P	O	K	P	O	K	P	O	K	P	O	K	P	O
1*	10	9	1	13	11	2	6	-	6				6	-	6	4	4	-	2	-	2	8	-	8	9	-	9			
2*	8	4	4	8	3	5	8	-	8				4	-	4	4	-	4				8	-	8	8	-	8			
3*	5	-	5	18	-	18	14	12	2	8	2	6	10	-	10	4	-	4	2	-	2	4	-	4	13	-	13	4	-	4
4*	4	-	4	4	-	4	4	-	4				4	4	-	8	8	-				8	-	8	12	-	12			
5*																4	4	-	4	-	4				4	-	4			
6*	1	-	1	2	-	2	2	1	1				2	-	2				1	-	1				1	-	1			
7*	1	-	1	2	-	2	2	-	2				2	-	2				2	-	2				1	-	1			
8*	4	-	4	8	-	8	8	-	8				16	-	16	4	-	4	9	-	9	8	-	8	20	-	20	4	-	4
9*	8	-	8	8	-	8	4	-	4	4	-	4	4	-	4	4	-	4				8	-	8	16	-	16			

Provisional designations: K -- number of experiments; P -- number of positive results; O -- number of negative results.

Antigens: 1* -- Typhoid "H"; 2* -- Typhoid "O"; 3* -- Tularemia; 4* -- Brucellosis Wright; 5* -- Brucellosis Heddelson; 6* -- Brucellosis vaccine (live, dried); 7* -- Morgan's bacilli; 8* -- Anthrax bacilli; 9* -- Enteric bacilli.

The preparations were prepared in the following manner. Onto scoured microscopic slides, using a large loop, drops of the mixtures of diagnostic antigens or of cultures with differing concentrations of microbial cells were placed; these were then dried at room temperature, after which the preparations were carefully fixed by flaming. Onto these smears, which were placed in moisture chambers made of Petri dishes, the same loop was used to add drops of the fluorescent sera, used in working dilutions. The moisture chambers with the preparations were then placed into the oven and kept there for varying periods of time (20 to 60 minutes), depending on the regime for one or another serum. After this, the preparations were placed for five to seven minutes in 0.1 milliliter buffer solution prepared from singly- and doubly-substituted sodium phosphate at a pH of 7.2 to 7.4; the smears were then rinsed with distilled water and dried in air. The preparations were studied with ordinary light microscopes, the source of ultraviolet and blue-violet rays being a Zeiss arc lamp for luminescent microscopy with a system of corresponding filters. The preparations were viewed under oil immersion, using an oil which was free of luminescence of its own. For orientation with respect to the number of bacterial cells in the preparations, comparable smears were made up, stained with Gram's stain, and studied under various magnifications with an ordinary light microscope.

Of 79 tests for the staining of antigens with homologous fluorescent antibodies, clearly positive results were obtained in 57 (Table 1). The bacterial cells, slightly enlarged in dimensions, shone with a specific bright yellow or yellowish-green light while retaining their characteristic morphology. Of the 22 negative results, 10 occurred as a result of low original concentrations of bacteria in the mixtures used (125 million or less per milliliter) and, just as in the case of the remaining negative results, are explained by our doubts concerning the luminescence of conglomerates and of separate cells with uncertain morphology, which we noted in the preparations. Of 321 tests of the processing of antigens with heterologous antibodies, only in five was there specific fluorescence of antigens, which may be explained as due to contamination during the processing.

The sensitivity of the method permitted, in several instances, a detection of the agents in concentrations of 62.5 million bacterial cells per milliliter of mixture, and unfailing demonstration of the agents in concentrations of 250 million bacterial cells per milliliter. When the microbe

cells were concentrated by centrifugation or filtration through membrane filters, the sensitivity of the method was increased accordingly.

A second series of tests was run to determine the possibility of detecting homologous antigens in environmental objects with the sera at hand. For this purpose, to 50 milliliters of distilled water and 10 milliliters of pond water were added 250 to 500 million cells of the appropriate antigen; 0.5 to one billion microbe cells were spread uniformly over 100 square centimeters of the surface of the object, and to 2.5 grams of food products placed in a Petri dish were added two milliliters of a one-billion-cell mixture of the antigen.

The bacterial cells mixed in distilled water were concentrated by filtration through a No 3 membrane filter; the residue was then suspended in one milliliter of distilled water and re-emulsified, then centrifuged for 15 minutes (at 5,000 revolutions), with subsequent decanting of the supernatant fluid and re-emulsification of the sediment in one milliliter of distilled water. The thickened mixtures thus obtained were then studied according to the method described above. It was observed that smears prepared from the filtrate contained 1.5 to 2.0 times more microbe cells than did smears from the centrifugate. From pond water heavily contaminated with extraneous particles, preparations were made up after preliminary purification. Purification was carried out by means of filtration or by allowing the water to stand for 10 to 15 minutes, with subsequent centrifugation (15 minutes at 1,500 revolutions). From the resultant supernatant fluid, or from the filtrate fluid, which contained up to 70 percent of the antigens introduced initially, along with the finest of the extraneous particles, the agents being sought were concentrated by filtration through a membrane filter and the preparations were then made up from the re-emulsified mixtures.

From the contaminated surfaces of objects, the introduced bacteria were removed with moistened tampons. In order to free the removed material of extraneous particles, we used the following method. From the contaminated surfaces of the objects, washings were made with a tampon moistened in water, with the tampon washed on each occasion with four to six milliliters of distilled water in a test tube, and then squeezed against the side of the test tube. The resultant turbid mixture was then processed by the same method used for the purification of contaminated water.

From the loose food products, fats, curds, and meat, preparations were made up in this fashion after freeing the

emulsions and washings from extraneous inclusions. The results of this series of tests for the detection of deliberately introduced antigens are shown in Table 2.

Table 2

Object of study	Antigen	No of tests	Results: Positive	Doubtful	Negative
Tap water	Brucellosis	6	6	-	-
	Tularemia	4	4	-	-
	Typhoid fever	4	4	-	-
Ground water (from pond)	Typhoid fever	14	13	-	1
Surface of a glass	Brucellosis	4	-	1	3
	Tularemia	2	-	1	1
	Typhoid fever	1	-	-	1
Surface of structures and equipment (worn from dust	Brucellosis	22	13	4	5
	Typhoid fever	23	18	1	4
Surface of concrete and other coverings (very dusty)	Typhoid fever	15	8	2	5
Surface of military material	Brucellosis	26	14	9	3
	Typhoid fever	26	16	7	3
Various grains	Brucellosis	5	3	2	-
	Typhoid fever	9	7	2	-
Butter	Brucellosis	2	2	-	-
	Typhoid fever	2	2	-	-
Combined fats	Brucellosis	5	-	2	3
	Typhoid fever	5	-	4	1
Frozen meat	Brucellosis	5	-	2	3
	Typhoid fever	5	-	2	3
Dried fruits	Brucellosis	2	-	2	-
	Typhoid fever	2	-	2	-
Curds	Brucellosis	2	1	1	-
	Typhoid fever	2	1	1	-

Note: In 12 experiments, structural surfaces were studied; in 26 experiments the surfaces of military material were studied; in 22 experiments with food products, the objects were contaminated with a mixture of brucellosis and typhoid fever diagnostic antigens. The experiments were accompanied by appropriate controls for specificity.

The "doubtful" results occurred in cases in which the antigens in the preparations fluoresced with a pale yellow color (++) and did not possess the characteristic morphology to a clear-cut degree.

Tests for the detection of deliberately introduced, pre-known bacteria confirmed the high specificity of the method and permitted, in the succeeding series of tests, a transition to the solution of the problem of detecting deliberately introduced but "unknown" agents in environmental objects.

We began the solution of the problem of detecting bacterial contamination of objects in the environment by procuring purified, concentrated mixtures of the agents being sought. For orientation in the choice of labeled sera necessary for the processing of the preparations, smears were made preliminarily from each of the concentrated mixtures and stains with Gram's stain. The characteristic morphology and staining properties of the corresponding agents permitted us to prepare the necessary number of smears and to stain them with one or another labeled serum.

Further processing of the preparations and study of them were carried out according to the method already described. Each test was accompanied by controls: No 1 -- deliberately introduced known antigens (determined by orientative microscopy), processed with homologous labeled sera; No 2 -- smears of the sought-for mixtures stained with normal serum. A third control was provided by smears of the sought-for mixtures stained with heterologous sera. For example, detected cocci were stained with brucellosis and tularemia sera, one of which, in the presence of bacteria of this morphology, was heterologous to them.

The preparation of smears, stained with labeled sera, from water containing a morphologically homogeneous species of bacteria, and microscopy of these smears, requires from 2.0 to 3.5 hours; approximately the same length of time is consumed in making up preparations from washing of objects. In microscopy of the preparation with the luminescent microscope, the bacterial cells and foreign particles are found in great abundance at the edge of the drop and thin out toward the center. The edge of the drop, if it contains bacteria which are homologous with the serum, shines brightly with a confluent and specific color. In the absence of homology between bacteria and serum, the edges of the drop fluoresce with a pale blue-gray color and show separate conglomerates of foreign particles, which shine with a bright yellow and yellow-orange color.

Since foreign particles do not form a confluent field of color, then the presence of such a confluent field, even in the absence of separate cells with clear-cut morphology, permits presumption as to the presence of the specific agent. Such luminescence of the edge of the drop (the preparation) necessitates a study of new preparations and of controls with different staining techniques. An identical picture in the first control testifies to the specificity of the luminescence and hence to homology between the bacteria and the serum. Simultaneously, it is noted that sera labeled with fluorescine-isocyanate, upon microscopy in the luminescent microscope, shine for a longer period, whereas sera labeled with 1-dimethylaminonaphthalene-5-sulfochloride quickly lose their capacity to fluoresce in the beam of ultraviolet rays.

Of 27 problems in the detection of bacterial contamination of the environment, 20 were solved correctly; in four cases, in addition to the detection of the introduced agent, others were discovered which were not a part of the problem; only in three instances were the introduced bacteria not detected. Hence, in evaluating the method only in terms of the solution of the problem of detecting unknown agents, it may be affirmed that the method is highly specific. In our material, 83 percent of the introduced agents were detected. Errors in the determination of specificity are explained by the absence of sufficient experience in the use of the method and, possibly, by nonspecific adsorption of antibodies on certain of the heterologous antigens. Experience in working with sera of higher specificity will permit increases in the number of positive results in studies using the fluorescent antibody method.

Conclusions

1. The highly specific and sensitive fluorescent antibody method is easily applicable to the quick detection of various agents.

2. Most convincing is the use of the method for detecting agents in water and in washings from slightly contaminated surfaces, in which the percentage of positive results approximates 100.

3. Satisfactory results have been obtained in the use of this method in studying heavily contaminated surfaces and certain food products. The agents of typhoid fever, brucellosis, and tularemia can be detected. Bacillary forms of bacteria with clear-cut morphology can readily be diagnosed. ( )